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ADAR1 interacts with PKR during HIV infection of lymphocytes and contributes to viral replication

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ABSTRACT

The interferon-induced protein kinase RNA-activated (PKR) is activated after virus infection. This activation is only transient during human immunodeficiency virus type 1 (HIV-1) infection of lymphocytes and the protein is not activated at the peak of infection. We observed that the interferon-induced Adenosine Deaminase Acting on RNA (ADAR)1-p150 and ADAR1-p110 expression increases while the virus replicates actively. Furthermore, both forms of ADAR1 show enhanced interactions with PKR at the peak of HIV infection, suggesting a role of this protein in the regulation of PKR activation. We observed that ADAR1-p150, as previously shown for the TAR RNA binding protein, TRBP, reverses PKR inhibition of HIV expression and production in HEK 293T cells. This activity requires the Z-DNA binding motif and the three double-stranded RNA binding domains, but not the catalytic domain. In astrocytic cells, ADAR1-p150 increased HIV expression and production to a similar extent as TRBP. Small interfering RNAs against ADAR1-p150 moderately decreased HIV production. These results indicate that two interferon-induced proteins, ADAR1 and PKR have antagonistic functions on HIV production. They suggest that ADAR1 and TRBP belong to a multi-protein complex that inhibits PKR during HIV infection of lymphocytes.

Keywords: HIV, PKR, ADAR1, TRBP, lymphocytes, astrocytes,

INTRODUCTION

Treatment of human cells by interferon (IFN) induces the expression of hundreds of IFN-stimulated genes (ISGs), some of which have an antiviral activity. These genes include the 2'-5'-oligoadenylate synthetase, Adenosine Deaminase Acting on RNA 1 (ADAR1), Mx GTPases, Major Histocompatibility Complex class I and II, protein kinase RNA-activated (PKR) and many others (47). Among the ISGs, PKR is a key serine/threonine kinase that has antiviral and antigrowth activities (14, 32). PKR is activated by dimerization after binding to low levels of dsRNA through its two double-stranded RNA binding domains (dsRBDs) (46). Once active, PKR phosphorylates a few substrates, among which the best characterized is the alpha subunit of the translation eukaryotic initiation factor 2 (eIF-2α), which negatively alters the efficiency and rate of translational initiation.

PKR activation is a critical component of antiviral and cell growth pathways (19) and its importance is illustrated by numerous cellular and antiviral mechanisms aiming to counteract its response. Viral mechanisms include the expression of competitive inhibitory RNAs or viral proteins that act either by direct inhibition of PKR, by sequestration of dsRNA, as competitive substrates or as translational rescuers by dephosphorylating eIF2α (19, 20). Cells also control PKR activation to limit the translational repression induced by the protein and to control cell growth. For example, the ribosomal L18, the TAR RNA binding protein (TRBP) and $p58^{IPK}$ sequester dsRNA or prevent PKR phosphorylation (20). Inhibition by protein-protein interactions also occurs with TRBP, tRNA-dihydrouridine synthase A and ADAR1, which bind PKR through their dsRBDs (16, 34, 35). In contrast, dsRNA, heparin and cellular proteins MDA7, PKR activator (PACT) and E2F-1 activate PKR (26, 37-40, 49). Viruses have also adapted to the cell in which they replicate by using cellular factors to regulate PKR activation. For example, Influenza virus activates $p58^{IPK}$ (31), Herpes virus US11 inhibits PACT (44), HIV TAR RNA recruits TRBP in proximity of PKR (13, 16, 36), and Vesicular Stomatitis virus (VSV) uses ADAR1 to inhibit PKR (35).

ADARs are RNA-editing enzymes that modify nuclear and viral RNAs by deamination which convert adenosines to inosines (6). Full-length ADAR1 enzymes possess two N-terminal Z-DNA binding domains (Z-DBD), three central dsRBDs, and a C-terminal deaminase domain. Three immunologically related isoforms of ADAR1 are found in human cells: the IFN-inducible cytoplasmic 150-kDa protein and constitutively expressed 110-kDa and 80-kDa proteins, which lack the first Z-DBD or both Z-DBDs plus the first dsRBD, respectively (50). The 150kDa form of ADAR1 was recently shown to bind to and inhibit PKR and to increase susceptibility to VSV infection (35). Whether ADAR1 plays a role as a PKR inhibitor in other viral infections has not been explored.

HIV expression is controlled at the transcriptional, post-transcriptional and translational levels (3, 21, 29). HIV-infected cells treated with IFN show a decreased production of HIV proteins and a reduced HIV production mainly ascribed to PKR activation (8). The HIV-1 Tat protein was shown to inhibit PKR activity by acting as a competitive substrate (30). Astrocytic cells represent an example of naturally HIV-resistant cells with high PKR activation. In these cells, TRBP is expressed in very low amounts and cannot counteract PKR activation induced by the virus (4, 5, 36). Therefore PKR activation can become a barrier to HIV replication, but the status of PKR phosphorylation has not been studied during viral infection of lymphocytes.

In this paper, we show that PKR is only transiently activated during HIV infection of lymphocytic cells. The analysis of cellular factors that interact with PKR during HIV infection shows that ADAR1 plays an important role in the inhibition of the kinase function during active replication.

MATERIALS AND METHODS

Plasmid constructions and siRNA synthesis.

pCMV-ADAR1 plasmid, containing ADAR1 mRNA (1 to 4058), GenBank accession # NM_001111.3, was obtained from Dr. K. Nishikura (12). This plasmid was used as a template to generate a cloning intermediate plasmid, ADAR1-p150 (1 to 3678), with a XhoI cleavage site added to the 3' site to facilitate cloning. ADAR1-p150 fragment was cleaved with HindIII and XhoI and subcloned into the pcDNA3.1_V5 vector (Invitrogen). This construct was used to generate the different variants ADAR-p110 (888 to 3678), ADAR p80 (1869 to 3678), ADAR p70 (1 to 1869), and ADAR Dcat (1 to 2475). All constructs were verified by sequencing. pGL2-LTR-Luc, pcDNA3-TRBP2, pcDNA1-PKR were previously described (16, 18, 33). Sequences of siRNAs used in this study were: NS (13), siA (35) or si4 (Qiagen SI00292320). They were all synthesized by Qiagen.

Cells and transfections.

Astrocytoma cell line U251MG (5) and HEK 293T (ATCC CRL-11268) were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). HEK 293T cells express adenovirus sequences and SV40 Large T antigen (41). Jurkat T cells (ATCC TIB-152) and Jurkat-CCR5 (2) obtained from Dr. K. Peden were maintained in RPMI-1640 (Invitrogen) supplemented similarly and with 0.4 mg/ml G418 (Multicell) for Jurkat-CCR5. For transfection of HEK 293T and astrocytes, cells were plated at 50% confluence 24 h prior to transfection using TransIT-LT1 Transfection Reagent following manufacturer's protocol (Mirus). Transfection of HEK 293T cells with siRNAs was performed in six-well plates using Lipofectamine 2000 (Invitrogen) as previously (13) 24 h prior to transfection with pNL4-3 using TransIT-LT1 (Mirus). Cells were lysed 48 h posttransfection for immunoblotting or luciferase analysis.

Transfection of HIV clones and reverse transcriptase (RT) assay

For transfection of HIV provirus, HEK 293T were transfected as above with pNL4-3 or pMAL proviral DNA. Cell supernatants were collected 48 h post transfection and assayed for standard RT assay (7) except that reaction was spotted onto DEAE filtermat (PerkinElmer). After 5 washes in 2X SSC, and twice in 95% ethanol, the filtermat was air-dried and read using Microbeta Scintillation counter (PerkinElmer). These supernatants were used for infection of Jurkat or Jurkat-CCR5.

HIV-1 viral infection.

For each infection, 10^7 Jurkat or Jurkat-CCR5 cells were infected with HIV cell supernatant corresponding to 2.5 x 10^6 cpm measured by standard RT assay in a final volume of 5 ml RPMI (Invitrogen), supplemented as above, and incubated for 2 h at 37°C with mixing every 30 min. 10 ml of RPMI was then added to the cell-virus mixture, transferred to a T75 flask and incubated overnight at 37°C. Another 15 ml of medium was added and the cell culture was maintained at 37°C for 25 days. The cells were fed every other day (or three days when their growth was not sufficient) by replacing 12 ml of supernatant with fresh medium and maintaining the cell density between 2.5 x 10^6 and 1 x 10^7 cells/ml. Supernatant and cell samples were collected at different times and assayed for RT activity, immunoblotting and immunoprecipitation (IP).

Immunoblotting

HEK 293T, U251MG or Jurkat T cells extracts were prepared, separated and transferred for immunoblotting as previously described (27). Membranes were blocked for 1 h in 5% nonfat dry milk and Tris-buffered saline-0.1% Tween 20 (TBST) or 5% BSA and 0.1% TBST for anti- PKR-pT^{451} antibody (Biosource). Membranes were incubated overnight at 4°C with the primary antibody. After

five washes in TBST, membranes were incubated with Horseradish Peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibody (GE Healthcare). Anti phosphorylated PKR (P-PKR) was used first in 3% BSA/TBST and the membranes were washed overnight in TBST and reused to detect other proteins. The bands were visualized using ECL (GE Healthcare). Primary antibodies used for immunoblotting in 5% milk/TBST were monoclonal anti-PKR 71-10 (28) obtained from Dr. A. Hovanessian at a 1/500 dilution, anti-GAPDH (Santa Cruz) at a 1/2500 dilution, anti-HIVp24 183-H12-5C (9), anti-V5 (Invitrogen) , anti-Actin (Chemicon) at a 1/5000 dilution, polyclonal anti-P-PKR, anti-human ADAR1 (a kind gift from Dr. B. Bass) at a 1/1000 dilution, and anti-TRBPjbx as previously (15). Actin or GAPDH was probed on each separate blot. Where indicated, the bands where quantified by densitometry analysis as described (17).

Immunoprecipitation

HIV-infected and mock-infected Jurkat T cells were washed twice with ice-cold PBS and lysed in the cold lysis buffer with protease inhibitors. For each IP, 50 µl of protein G agarose fast flow compact beads (Sigma) were washed with ice-cold PBS and left rotating at 4°C for 4 h with 8 µg anti-PKR 70-10 or 5 µg anti-human ADAR1 antibodies. 2.5 mg of cell extracts were added to the beads for overnight incubation at 4°C. The beads were washed 5 times with 1 ml of ice-cold PBS and resuspended in SDS loading dye. Bound proteins were eluted by boiling the beads for 5 min and fractionated by 10% SDS-PAGE. The immunoprecipitates were analyzed by Western blot analysis using appropriate antibodies.

RESULTS

PKR activation is inhibited during active HIV replication.

PKR becomes activated after transfection of HIV molecular clones in non-productively infected astrocytes, but not in productively infected HeLa cells (36). To determine if PKR becomes activated in lymphocytes, we analyzed its phosphorylation during viral infection. Jurkat cells were infected by HIV pNL4-3, a strain that uses CXCR4 as a coreceptor (1, 43) and viral kinetics were followed by RT assay on the culture media over 25 days (Fig. 1A). Cell extracts were analyzed for PKR expression and phosphorylation (Fig. 1B). We observed that PKR was transiently phosphorylated up to day 6. This phosphorylation decreased at day 8-10 and was no longer observed after day 12. This PKR activation correlated with the appearance of RT activity that became visible at day 10 and showed a peak at day 11 to 15, corresponding to active viral expression and production as shown by immunoblotting against HIV p24 antibody revealing $p55^{GAG}$ expression (Fig. 1B). This correlation suggests that PKR activation is inhibited during active viral replication.

Because TRBP has been demonstrated to be a strong inhibitor of PKR in the context of HIV expression (8, 13, 16, 36), we verified if an increase of its expression could explain the lack of PKR activation, but no correlation between TRBP levels and PKR activation was observed (Fig. 1B). ADAR1 is also a PKR inhibitor in HEK 293T cells and during VSV infection (35) and therefore its expression was verified on the same extracts. Surprisingly, a strong increase of both ADAR1 p110 and p150 forms correlated with the appearance of HIV $p55^{GAG}$ expression and the decrease of P-PKR (Fig. 1B). Cell extract analysis from a mock infection of Jurkat cells performed in the same conditions showed no PKR activation, indicating that besides HIV infection, no other parameter has influenced PKR activation (Fig. 1C).

pMAL is an HIV strain that uses CCR5 as a coreceptor, and can replicate in lymphocytes that express the appropriate coreceptor (42, 43). We infected Jurkat-CCR5 cells (2) with pMAL and

followed viral kinetics (Fig. 1A). RT assays show an overall lower activity compared to pNL4-3 in this settings. Viral production peaked at day 15 and was half the production of that of pNL4-3. pMAL-infected Jurkat cell extract analysis showed that PKR was phosphorylated up to day 9, then remained weakly activated throughout the infection (Fig. 1D). In this infection, TRBP and ADAR1 p150 were moderately increased after day 13, suggesting a contribution of both cellular proteins. The calculated P-PKR/PKR ratio measured during the infection by pNL4-3 and pMAL reflected an overall decrease of PKR activation during active HIV replication (Fig. 1E)

HIV-infection of lymphocytes increases ADAR1-PKR interactions.

Because we observed an increase in ADAR1-p150 and p110 expression during HIV infection of Jurkat cells we evaluated whether the interaction of these proteins with PKR reflected these modifications. Cell lysates from mock-infected and HIV pNL4-3 infected Jurkat T-cell at the peak of infection were immunoprecipitated with an anti-PKR antibody and the associated proteins were analyzed using antibodies against PKR, ADAR and TRBP (Fig. 2). Whereas TRBP-PKR interactions were not changed by viral infection, we observed a dramatic increase in binding of both the cytoplasmic full-length and the nuclear spliced form of ADAR1 to PKR in the presence of replicating HIV. This result suggested a role of the protein in enhancing HIV replication by controlling PKR activation. Furthermore, the reverse IP with the ADAR antibody showed that both ADAR1-p150 and p110 variants, PKR and TRBP were immunoprecipitated and ADAR-PKR interactions were also increased in the presence of HIV (Fig. 2, upper right panel).

ADAR1p150 reverses PKR-inhibition of HIV-1 LTR expression and viral production

Because ADAR1 has been shown to inhibit PKR activation (35) and because it may have a similar role during HIV replication, we verified if the protein was able to reverse PKR inhibition of

HIV long terminal repeat (LTR) expression. A similar role has been previously attributed to TRBP (16) and we therefore compared ADAR1 and TRBP in the same assay (Fig. 3A). In this context, both ADAR1 and TRBP reversed the PKR-mediated inhibition of HIV-1 LTR, suggesting a similar activity. ADAR1 was also compared to TRBP in the context of HIV-1 production (Fig. 3B). As previously, transfection of a PKR expressing vector inhibited HIV expression in HEK 293T cells and TRBP reversed this effect (36). In this assay, ADAR1 had the same activity as TRBP, strongly suggesting that ADAR1 is also a cellular inhibitor of PKR during HIV replication.

ADAR1-p150 increases HIV production in the presence and in the absence of overexpressed PKR

To adequately monitor the expression and the activity of ADAR1-p150 and compare it with mutant forms, we constructed a plasmid expressing a tagged protein with V5 in its C-terminus. We first determined if this tagged protein had the same activity as the untagged form for PKR inhibition in the context of active HIV production. HEK 293T cells were transfected with PKR and pCMV-ADAR1-p150 or pcDNA3-ADAR1-p150-V5 (Fig. 4). Cell culture supernatants and lysates were collected and assayed for RT activity and protein expression, respectively. In the context of a ten-fold inhibition of HIV expression by PKR, the addition of either form of ADAR-p150 reversed this inhibition completely and added an additional twofold increase above the original level. This result indicates that the long form of ADAR1 is a powerful PKR inhibitor, whether it is tagged or not (Fig. 4A). The expression of HIV $p55^{GAG}$ protein confirmed the restoration of viral protein production with ADAR1 (Fig. 4B). Unexpectedly, although we transfected the same amount of PKR expressing vector, PKR expression increased with ADAR1 plasmid transfection. To determine if part of the increased HIV production could be ascribed to a PKR-independent activity, the same experiment was performed with ADAR1-V5 in the absence of exogenous PKR. In this case, a maximum of a twofold

increase in HIV production was observed with increasing amounts of ADAR1-p150-V5, with a slight decrease at the highest concentration (Fig. 4C). We noted that increased ADAR1 concentrations had no effect on endogenous PKR expression.

ADAR1 inhibition of PKR requires the three dsRBDs and is not due to deaminase function

To test which part of ADAR1 is responsible for inhibition of PKR, full-length ADAR1 (p150), the truncated variants p110 and p80 (50), a mutant deleted in the catalytic deaminase function (Dcat) and a mutant with only the Z-DBDs and dsRBD1 (p70) were tagged with the V5 epitope at their C-termini and were transiently transfected into HEK 293T cells (Fig. 5A). To determine the ability of the ADAR1 variants and mutants to reverse PKR activity, they were first assayed in a HIV1-LTR luciferase assay as in Fig 3A (Fig. 5B). In conditions where PKR inhibited the LTR activity fivefold, ADAR1-p150 restored this activity almost completely, Dcat increased it threefold relative to PKR only, p110 increased it by less than twofold relative to PKR only, but ADAR p80 and p70 mutants failed to restore luciferase expression. The wild-type and mutants were then tested on the rescue of HIV production inhibited by PKR (Fig. 5C). In this case, HIV production was fully restored by ADAR1-p150 whereas a threefold and twofold increase relative to PKR only was observed with Dcat and p110 respectively. Similar to the above results, p80 and p70 had no or very mild activity in this context. These results were confirmed by immunoblotting against HIV p24, which showed a complete rescue of viral protein expression with ADAR1-p150 and a partial one with Dcat and p110. The expression of the different ADAR1 variants and mutants show that they were all expressed, but they exhibit some variations in their level of expression. Although the full-length p150 form was weakly expressed, it had the strongest activity, suggesting that its real activity is much more potent than the one seen in the luciferase and RT assays. Overall, these results suggest that the deaminase function is not required for PKR inhibition, but that the integrity of the three dsRBDs is necessary.

It was recently suggested that overexpression of ADAR1 or ADAR2, another member of the ADAR family, increases HIV production in the absence of PKR transfection and this activity was ascribed to the deaminase function (45). To determine if it is also the case in our assay, we verified the activity of our constructs in the absence of transfected PKR (Fig. 5D). We observed a twofold increase in HIV $p55^{GAG}$ expression and only a mild increase (30-40%) in RT activity with wild-type ADAR1 and ADAR1 mutants. This increase occurred also with the mutant deleted in the catalytic domain, suggesting that the deaminase function does not affect HIV production in our assay. As in Figure 3 and 4, transfection of the various ADAR1-expressing plasmids increased the expression of transfected PKR (Fig. 5C), but not endogenous PKR (Fig. 5D).

ADAR1 increases HIV-1 production in astrocytes

Astrocytes are a model in which HIV replication is very low, due in part, to a high level of PKR activation that prevents viral translation (22, 36). This high PKR activation is due to the weak activity of the TRBP promoter. This induces the production of only very small amounts of TRBP, which are unable to counteract PKR activation (4, 5). To determine if ADAR1 also contributes to PKR inhibition in this cellular context, we analyzed the activity of ADAR1-p150-V5 compared to TRBP on HIV expression and production in the U251MG astrocytic cells (Fig. 6). In these cells, ADAR1 and TRBP induced up to threefold increases in HIV production and a similar increase in $p55^{GAG}$ expression. This result is compatible with ADAR1 activity as an inhibitor of endogenous PKR activation.

Inhibition of ADAR1-p150 expression decreases HIV expression.

A previous study showed that a siRNA against ADAR1-p150 only (siA) decreased the long form of the protein and decreased VSV production correlated with increased PKR activation (35).

Another study showed that a siRNA against both ADAR1-p150 and p110 decreased HIV expression but was not correlated to PKR activation (45). To further determine the role of ADAR1 in HIV replication, we used siA and a siRNA targeting both forms (si4, Qiagen), and analyzed their effect on viral expression and production (Fig. 7). The activity of siA mildly decreased ADAR1-p150, whereas si4 decreased both forms. Despite the mild activity of siA, virus expression was significantly decreased in cells and virus production was decreased by 30%. In contrast, si4 reduced HIV gag expression moderately and HIV virus production very weakly.

DISCUSSION

Although IFN is able to inhibit HIV production in cell culture (8), the IFN produced in plasmocytoid dendritic cells during HIV infection does not eliminate the virus in patients and, in long-term, contributes to pathogenesis (23-25, 48). This *in vivo* inefficacy could be due to an inadequate innate immune response during the first days of infection, but the activity of the ISGs on virus replication in lymphocytes during this time frame has been poorly investigated. Because PKR is one of the main ISGs that can inhibit HIV production in cell culture, we wanted to determine if PKR is activated or not during HIV infection of lymphocytes. We found that PKR becomes phosphorylated soon after HIV infection. This is followed by an inactivation of PKR, which correlates with HIV replication (Fig. 1). These results suggest that the innate immune response mediated by PKR is fully functional but is only transiently active. Because in astrocytes PKR activation is an important barrier to HIV expression and replication (36), we thought that HIV might specifically replicate in cells where PKR activation is repressed.

We studied the expression of ISGs and the role of various PKR inhibitors, and observed that the expression of ADAR1-p150 and p110 forms is enhanced during HIV infection, which correlates with increased HIV replication and increased PKR binding (Fig 1 and 2). Because ADAR1 is an ISG,

one could expect that this protein would contribute to a cell response against virus replication. While these results were being collected, ADAR1 was isolated in a two-hybrid screen using PKR as bait (M. Bonnet and E. Meurs, data not shown) and ADAR1 was shown as a PKR inhibitor in the context of VSV infection (35). Both studies showed that the first dsRBD in ADAR1 is the domain that binds PKR. Results of the two-hybrid screen showed that the five isolated clones all express amino acids 503 to 556 within dsRBD1. Similarly to TRBP, ADAR1 was able to reverse PKR inhibition in cells expressing only HIV LTR or with active HIV replication, indicating that its increased binding to PKR at the peak of HIV infection contributes to the enhancement of HIV replication in lymphocytes (Fig. 3 and 4A). The analysis of ADAR variants' and mutants' activity further demonstrates that the integrity of the three dsRBDs and at least one Z-DNA binding domain, but not the catalytic domain, are necessary for PKR inhibition (Fig. 5).

Because PKR is not expressed from an overexpressed plasmid in HIV-replicating cells, we evaluated the activity of ADAR1 in the absence of exogenous PKR. In this context, ADAR1 p150 increased HIV expression and production but this effect was only about twofold (Fig. 4C and 5D). A similar activity was recently observed in an independent study and showed that the deaminase function was responsible for this increased HIV production (45). However, we observed similar results with our Dcat mutant and ADAR1-p150 in the absence of PKR, which does not support the same conclusion (Fig. 5D). In addition, the activity of the Dcat in the presence of exogenous PKR (Fig. 5B and C) suggests a main activity by inactivating also endogenous PKR. The discrepancy between this previous study and our results is currently unexplained but could be due to a difference in experimental settings. Similar to the previous study, siRNAs that decreased both ADAR1-p110 and p150 decreased HIV expression, but this effect was stronger with a specific inhibition of the p150 form (siA in Fig. 7).

The similar activities of ADAR1 and TRBP in increasing viral production in astrocytes further suggests that ADAR1 could replace TRBP in regard to PKR inhibition and enhancement of HIV production in astrocytes (Fig. 6). The similar effect of siRNAs against ADAR1 (Fig. 7) and siRNAs against TRBP (13) confirms this hypothesis. Taken together, our results suggest that the main activity of IFN-induced ADAR1 p150 isoform is to counteract PKR inhibition of HIV expression. They indicate that two IFN-induced proteins can have opposite effects, which ultimately contributes to the enhancement of HIV replication in lymphocytes.

Previous results have shown that the virus itself can counteract PKR activation with its Tat protein that acts as a competitive substrate (10, 11, 30). Taken together, previous and present results suggest that PKR inhibition during HIV replication is mediated both by the viral Tat protein and by host factors ADAR1 and TRBP (Fig. 8). We have also shown recently that in addition to direct PKR inhibition, TRBP binds to and inhibits the PKR activator PACT (15, 27). This latter function could further contribute to PKR inhibition during HIV replication. It is also possible that in the experiments that use HEK 293T cells, the presence of VAI RNA and SV40 large T antigen also contributes to PKR inhibition (Fig. 3-5, 7), but this presence did not prevent the PKR and ADAR1 activities observed here. Overall, HIV uses at least three different mechanisms to counteract PKR activation: i) it produces the Tat protein during the early steps of its replication, ii) it has evolved to replicate specifically in cells that express high levels of TRBP and iii) it increases the synthesis of ADAR1-p150 isoform either directly, or indirectly, through IFN induction. Further studies of PKR-binding factors during HIV infection may reveal additional proteins that could contribute to PKR inactivation and enhanced virus replication.

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FIGURE LEGENDS

Figure 1: PKR is transiently activated after HIV infection and inhibited during active HIV replication. A) HIV-1 pNL4-3 and pMAL infection kinetics. Jurkat cells were infected with none (dotted line) or HIV pNL4-3 (grey line). Jurkat-CCR5 cells were infected with HIV-1 pMAL (black line). Aliquots of cell supernatants were collected at different times and assayed for RT activity. **B) Protein expression of pNL4-3-infected Jurkat cells.** (upper part) 250 µg of whole-cell extracts from pNL4-3-infected Jurkat cells were subjected to a 10% SDS PAGE and blotted with anti-P-PKR, anti-PKR, anti-HIV-p24 and anti-actin antibodies as indicated. (middle part) 250 µg of the same extracts were subjected to a similar SDS PAGE and blotted with anti-TRBPjbx and anti-actin antibodies as indicated. (lower part) 250 µg of the same extracts were subjected to a 7.5% SDS PAGE and blotted with anti-ADAR1 and anti-actin antibodies as indicated. Exposure was 10 times longer for ADAR1-p150 than for ADAR1-p110 + p150 when indicated. **C) Protein expression of mock-infected Jurkat cells.** 250 µg of whole-cell extracts from mock-infected Jurkat cells were subjected to a 10% SDS PAGE and blotted as in B with the indicated antibodies. **D) Protein expression of pMAL-infected Jurkat-CCR5 cells.** 250 µg of whole-cell extracts from pMAL-infected Jurkat-CCR5 cells were subjected to a 10% and a 7.5% SDS PAGE and blotted as in B with the indicated antibodies. **E) Ratio of phosphorylated PKR versus PKR during HIV infection.** The band intensity was digitalized using Adobe Photoshop software from the shown bands in B for pNL4-3 and in D for pMAL. P-PKR/PKR ratio was calculated by dividing the P-PKR intensity by the total PKR intensity of each band.

Figure 2: ADAR1-PKR interaction increases during HIV-1 infection. Jurkat cells were mock infected or infected with HIV pNL4-3. Cell lysates collected at day 15 (peak of infection) were immunoprecipitated with anti-PKR or anti-ADAR1. 250 µg of proteins from each lysate (input; lanes

1-2) and the PKR (lanes 3-4) or ADAR (lanes 5-6) immunoprecipitated complexes were run on a 10% SDS-PAGE and blotted using anti-PKR, anti-ADAR1, anti-TRBPjbx, anti-HIV-p24 and anti-actin.

Figure 3: ADAR1 p150 and TRBP2 reverse PKR inhibition of HIV expression and virus production. A) ADAR1 p150 and TRBP2 reverse PKR inhibition of HIV LTR expression. HEK 293T cells were transfected with 0.05 µg of pGL2-LTR-Luc (lanes 2-5), 0.5 µg of pcDNA1-PKR (lanes 3-5), and with 1 µg pCMV-ADAR1-V5 p150 (lane 4) or 1µg pcDNA3-TRBP2 (lane 5). Empty plasmids pcDNA1 and pcDNA3.1_V5 or pcDNA3 were added to reach the same amount of transfected DNA. % Luciferase activity is the ratio between the luciferase level in the presence of PKR and either ADAR1 or TRBP2 versus LTR-Luc alone. Shown is the average of 4 independent transfections ± SEM. **B) ADAR1 and TRBP reverse PKR-inhibited HIV-1 expression.** HEK 293T cells were transfected with 2 µg pNL4-3 (lanes 2-5), 0.5 µg pcDNA1-PKR (lanes 3-5) and 1.5 µg of pCMV-ADAR1 (lane 4) or pcDNA3-TRBP2 (lane 5). Shown is the average of 4 independent transfections ± SEM. (Top) RT assay from cell supernatants normalized to 100% in the absence of PKR or dsRBPs. (Bottom) 250 µg of each cell extract was analyzed by immunoblot against HIV p24, ADAR1, TRBP, PKR, or GAPDH as indicated. TRBP was blotted before HIV p24 and appears on the same blot. GAPDH was used instead of actin, which runs close to TRBP.

Figure 4: ADAR1-p150 increases HIV production in the presence and in the absence of overexpressed PKR. A) ADAR1 and ADAR1-V5 activity on PKR-inhibited virus production. HEK 293T cells were transfected with none (lane 1), 2 µg pNL4-3 (lanes 2-6), 0.5 µg pcDNA1-PKR (lanes 3-6), 0.5 µg (lane 4), 1.0 µg (lane 5) or 1.5 µg (lane 6) of ADAR1-p150-V5 (light grey) or ADAR1-p150 (dark grey). 48 h posttransfection, supernatants were collected for RT assay and cell lysates were generated. Shown is the average of 4 independent transfections ± SEM. **B) ADAR1 and**

ADAR1-V5 activity on PKR-inhibited HIV protein expression. 250 µg of cell extracts produced in A) without and with ADAR1 p150-V5 (lanes 1-6) or ADAR1 p150 (lanes 7-12) were subjected to 10% SDS PAGE and blotted with anti-HIV-p24, anti-ADAR1, anti-V5, anti-PKR and anti-actin antibodies as indicated. **C) ADAR1-V5 activity on HIV expression and virus production.** HEK 293T cells were transfected with none (lane 1), 2 µg pNL4-3 (lanes 2-5), 0.5 µg (lane 3), 1.0 µg (lane 4) or 1.5 µg (lane 5) of ADAR1-p150-V5. 48 h posttransfection, supernatants were collected for RT assay (top) and cell lysates were generated. Shown is the average of 3 independent transfections ± SEM. 250 µg of cell extracts were subjected to 10% SDS PAGE and blotted with anti-HIV-p24, anti-ADAR1, anti-V5 and anti-actin antibodies as indicated.

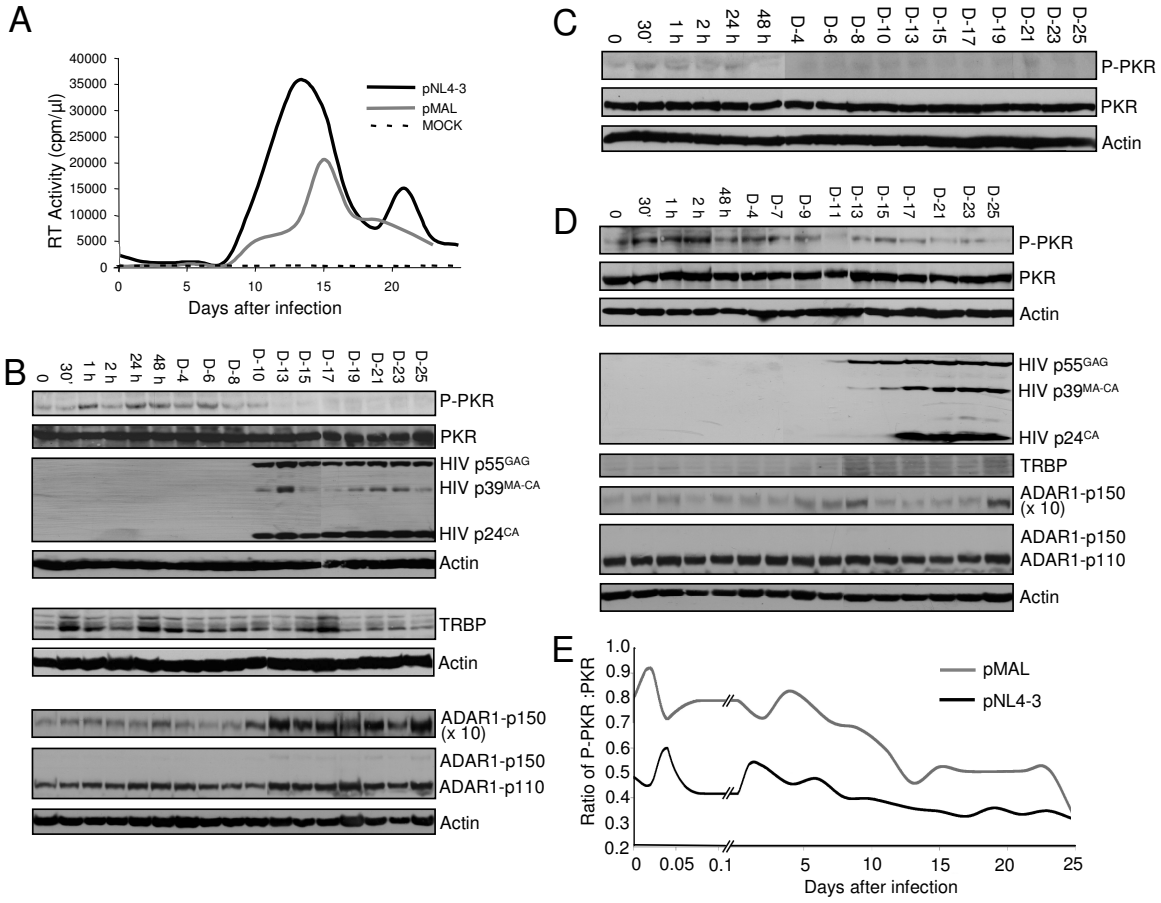
Figure 5: ADAR1 inhibition of PKR requires the dsRBDs but not the deaminase function. A) Schematic of naturally existing variants and mutant forms of ADAR1 tagged with V5. ADAR1-p150 is the full-length protein (aa 1 to 3678). Mutants and variants are ADAR1 Dcat (aa 1 to 2475), p110 (888 to 3678), p80 (1869 to 3678) and p70 (1 to 1869). The DNA-binding domain (Z-DBD), dsRBDs, the catalytic domain (Cat) and the V5 tag are indicated. **B) Activity of ADAR1 and ADAR1 mutants on PKR inhibited HIV-1 LTR expression.** HEK 293T cells were transfected with 0.10 µg of pGL2-LTR-Luc (lanes 2-8), 0.10 µg of pcDNA1-PKR (lanes 3-8), and with 1 µg ADAR1 p150 (lane 4), Dcat (lane 5), p110 (lane 6), p80 (lane 7) and p70 (lane 8). Empty plasmids pcDNA1 and pcDNA3.1_V5 were added to reach the same amount of transfected DNA. % Luciferase activity is the ratio between the luciferase level in the presence of PKR and different ADAR1 mutants versus LTR-Luc alone. Shown is the average of 3 independent transfections ± SEM. **C) Activity of ADAR1 and ADAR1 mutants on PKR inhibited HIV-1 production.** HEK 293T cells were transfected with 2.0 µg of pNL4-3 (lanes 2-8), 0.50 µg of pcDNA1-PKR (lanes 3-8) and with 1.5 µg ADAR1 p150 (lane 4), Dcat (lane 5), p110 (lane 6), p80 (lane 7) and p70 (lane 8). Empty plasmids pcDNA1 and

pcDNA3.1_V5 were added to reach the same amount of transfected DNA. (Top) % RT activity is the ratio between the RT level in the presence of PKR and different ADAR1 variants versus pNL4-3 alone. Shown is the average of 5 independent transfections ± SEM. (Bottom) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against V5, HIV p24, PKR and actin. **D) Activity of ADAR1 and ADAR1 mutants on HIV-1 production.** HEK 293T cells were transfected with 2.0 µg of pNL4-3 (lanes 2-7) and with 1.5 µg ADAR1-p150 (lane 3), Dcat (lane 4), p110 (lane 5), p80 (lane 6) and p70 (lane 7). Empty plasmids pcDNA1 and pcDNA3.1_V5 were added to reach the same amount of transfected DNA. (Top) % RT activity is the ratio between the RT level in the presence of different ADAR1 variants versus pNL4-3 alone. Shown is the average of 3 independent transfections ± SEM. (Bottom) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against V5, PKR, HIV p24, and actin.

Figure 6: ADAR1 and TRBP increase HIV-1 virus production in astrocytes. A) ADAR1 and TRBP increase pNL4-3 virus production in astrocytes. U251MG cells were transfected with none (lane 1), 2 µg pNL4-3 (lanes 2-5) and 0.5 µg (lane 3), 1.0 µg (lane 4) or 1.5 µg (lane 5) of pCMV-ADAR1-p150-V5 (light grey) or pcDNA3-TRBP2 (dark grey). Empty corresponding plasmids were added to reach the same amount of transfected DNA. % RT activity is the ratio between the activity in the presence of pNL4-3 and ADAR1 or TRBP versus pNL4-3 alone in the cell supernatant. Shown is the average of 4 independent transfections ± SEM. **B) ADAR1 and TRBP increase HIV protein expression in astrocytes.** 250 µg of cell extracts produced in A) without and with ADAR1-p150-V5 (lanes 1-5) or TRBP2 (lanes 6-10) were subjected to 10% SDS PAGE and blotted with anti-HIV-p24, anti-ADAR1, anti-TRBP, anti-PKR and anti-actin antibodies as indicated.

Figure 7: Decrease of ADAR1 p150 expression affects HIV production. HEK 293T cells were not transfected (lane 1) or transfected with none (lane 2), 14 nM of siNS (lane 3), siA (lane 4) or si4 (lane 5). They were transfected 24 hr later with 2.0 µg of pNL4-3 (lanes 2-5). (Top) RT activity of the cell supernatant is represented on the graph. (Bottom) Immunoblot of 150 µg of cell extracts from the same transfected cells using antibodies against ADAR1, HIV p24, and actin.

Figure 8: Schematic representation of the regulation of HIV translation by PKR and the contribution of host and viral factors. The viral TAR RNA contributes to PKR activation by inducing its phosphorylation, which in turn phosphorylates eIF2α and consequently inhibits HIV translation. During HIV replication, the cellular proteins TRBP and ADAR1 prevent or inhibit PKR phosphorylation whereas the viral protein Tat prevents eIF2α phosphorylation. All three proteins contribute to increased HIV translation.



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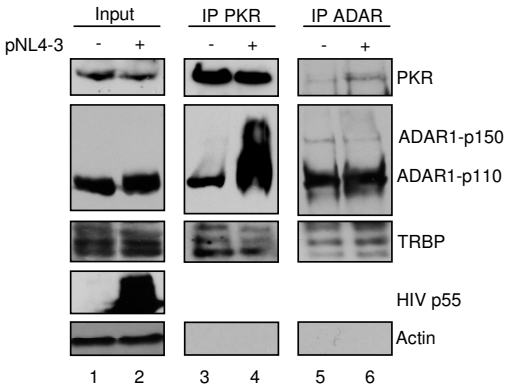

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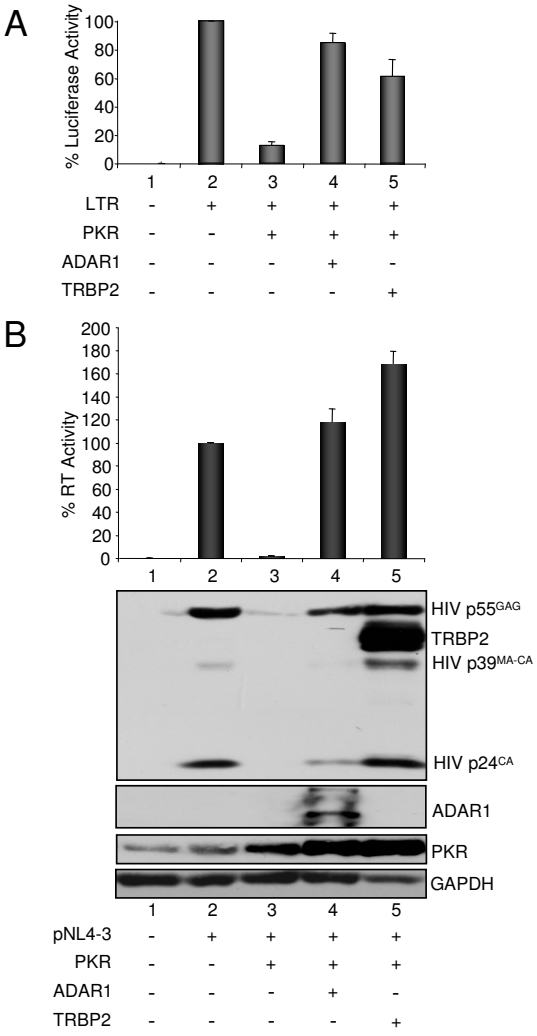

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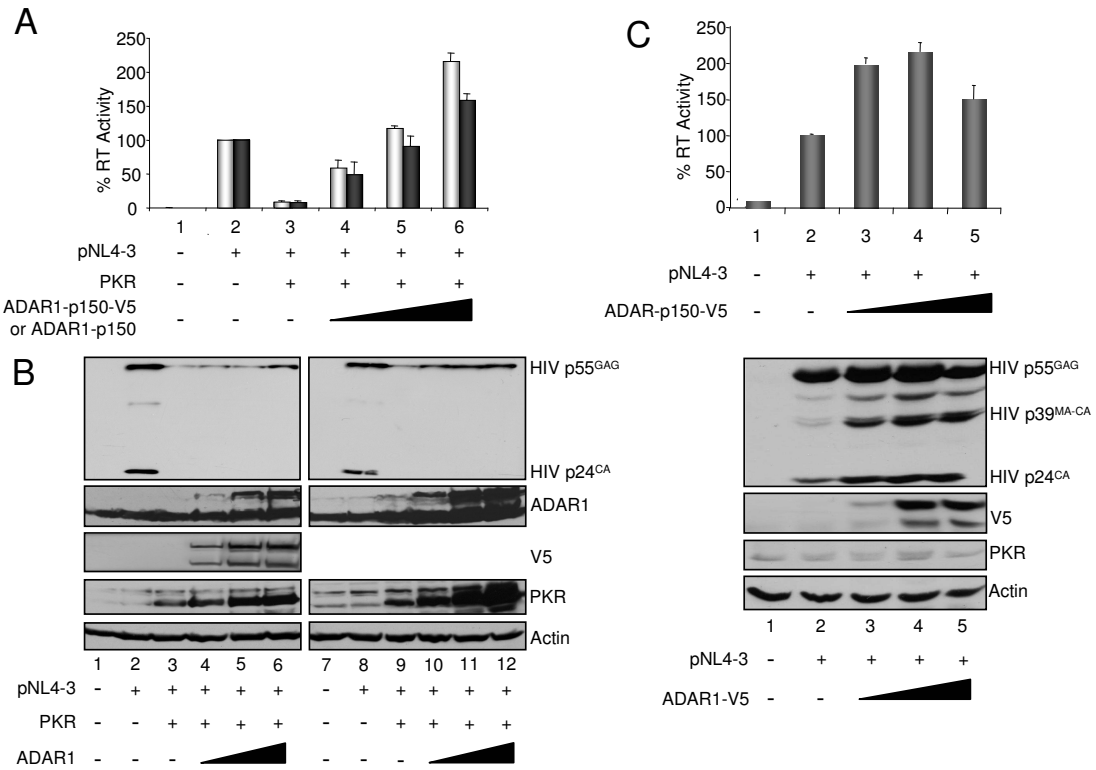

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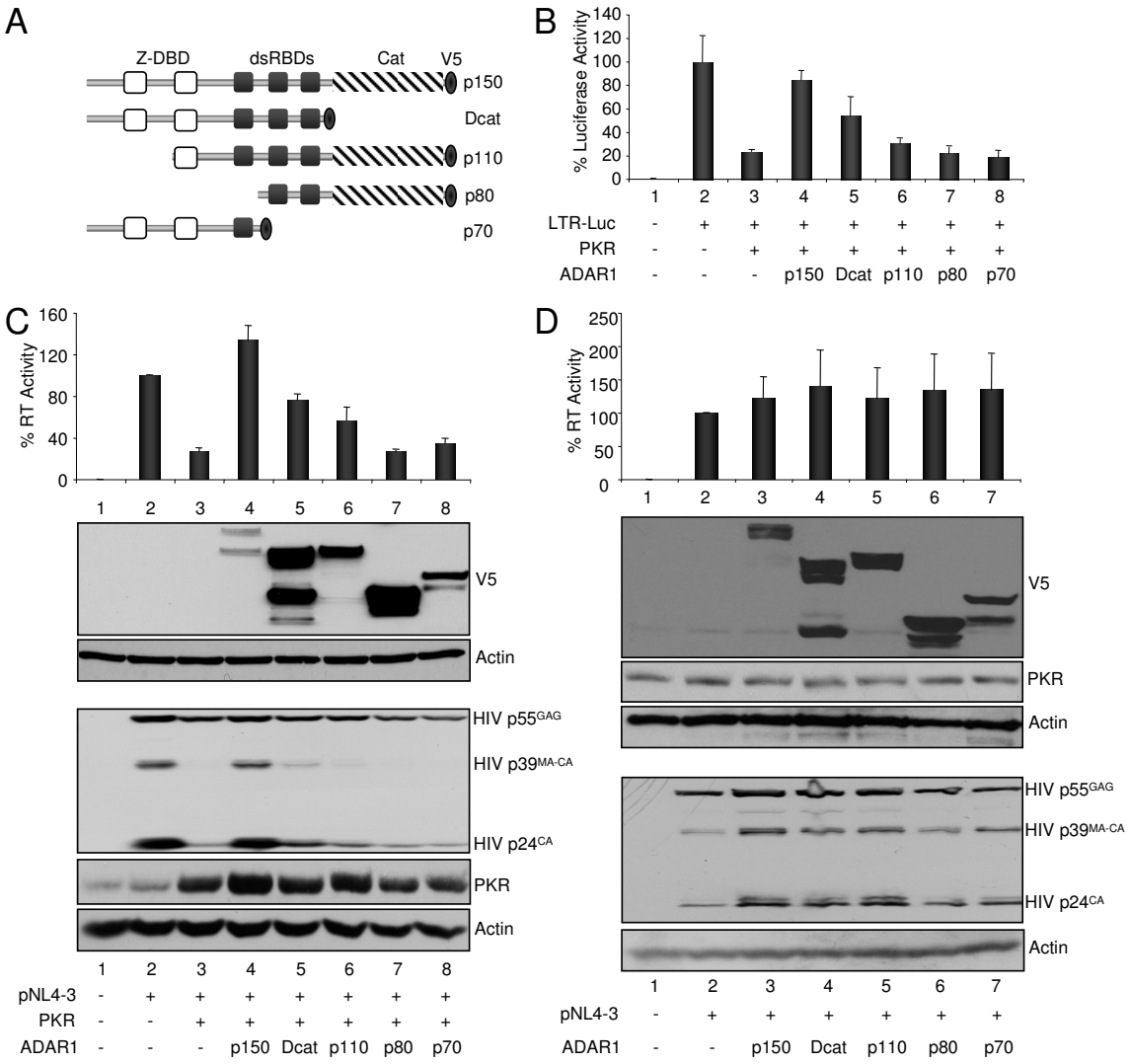

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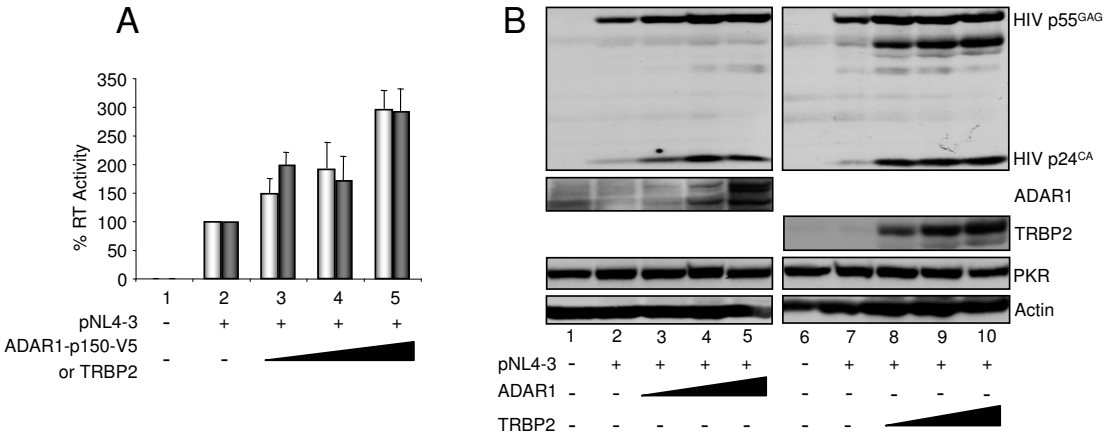

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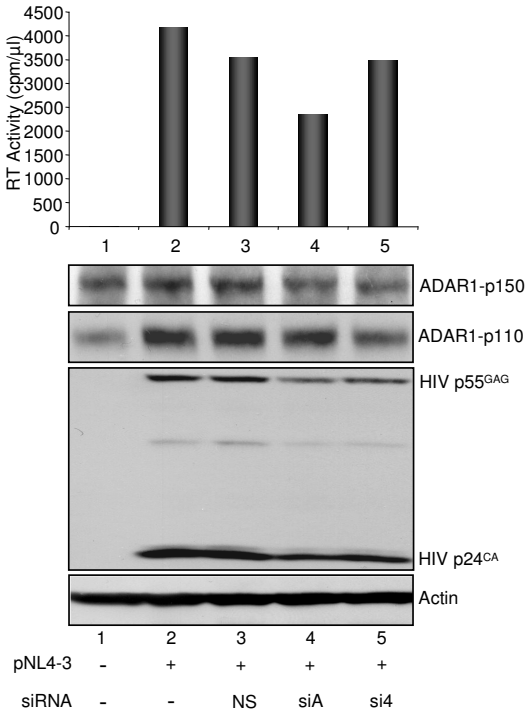

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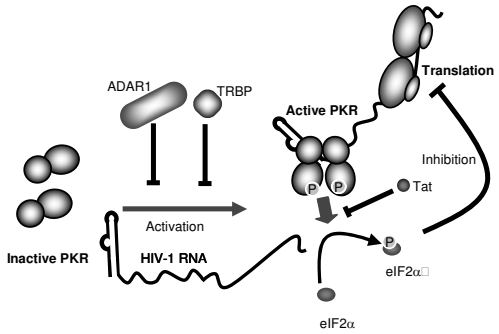

Clerzius, Fig. 8